SREENING METHOD FOR APOPTOSIS AND NECROSIS

Field of the Invention

5

The present invention relates to a method for the determination of non-, anti-, or pro-apoptotic and necrotic conditions of cells, using newly designed vectors coding for marker proteins, cell lines transfected
10 with such vector, and a method to assay the non-, pro-, or anti-apoptotic or necrotic activity of test compounds or physical stimuli.

15 ### Background Art

Apoptosis plays an essential role in development, i.e. embryogenesis and normal cell turnover, but also in diseases such as cancer, AIDS, neurodegeneration
20 and viral infections. Unlike necrosis, apoptosis is an active, gene-directed self-destruction process of the cell and is associated with characteristic morphological and biochemical changes [1, 2]. Nuclear and cytoplasmic condensation and fragmentation of the dying cell into
25 membrane-bound apoptotic bodies are typical characteristics of apoptosis. Another feature of apoptotic cell death is the chromosomal DNA degradation into oligonucleosomal fragments after the activation of specific nucleases [3, 4].

30 Apoptosis can be induced by the interaction of the cell surface molecule Fas (CD95) with Fas-Ligand (FasL), where the Fas expressing and sensitive cells undergo apoptosis. Fas is a type I membrane protein, which belongs to the tumor necrosis factor (TNF) and nerve
35 growth factor (NGF) receptor family [5-7]. Fas expression is found on a wide variety of tissues and cells such as thymus, liver, lung, ovary, heart and myeloid cells [9-12]

FasL expression is not only found on lymphocytes but also on a wide variety of tissues and some tumors 13-19. Both membrane bound FasL and the soluble form (sFasL) can induce apoptosis in Fas positive and sensitive cells. Other

5 forms among a variety of apoptosis mediators include the Perforin/Granzyme system, the TRAIL/TRAIL-R system 36, cytokine deprivation (e.g. IL-3 deprivation) and irradiation.

In contrast to apoptosis, necrosis is a non-

10 physiological death of cells due to chemical or physical injury of the cell membrane. Morphological criteria include cell swelling and cell lysis, lysosomal leakage and loss of the cell membrane integrity.

During the last decade, it has become clear

15 that apoptosis plays a keyrole in several diseases. Apoptosis is increased in AIDS, but decreased in cancer and certain autoimmune proliferative diseases.

Flow cytometry offers a wide variety of possibilities to measure apoptosis. Different methods have

20 been established and implemented, some which stain on the cell surface and some which stain intracellularily.

One of the first approaches was, beside the observation that apoptotic cells shrink and have higher intracellular granularity, to stain with DNA specific

25 fluorochromes (eg. propidium iodide [PI], ethidium bromide [EtBr]). As soon as a lethal hit is being induced, the DNA starts to change its profile. Apoptotic DNA not only consists of fragmented DNA (visualised as shorter bands, so called DNA ladder, in an agarose gel) but is

30 also partially digested into single nucleotides, so that fluorochromes, like PI or EtBr, have less DNA to stain. This is typically observed by a shift to the left, called sub-G1 peak, on the specific fluorochrome detection channel in the FACScan ™ (from Becton Dickinson, USA). The

35 big disadvantage of this method is that the cell membranes have first to be permeabilized with reagents, like ethanol, in order to stain them with DNA specific dyes,

like PI [20]. The treatment is time and labour consuming, and the risks of loosing cells and of handling errors are high. Furthermore, the discrimination between live and apoptotic cells cannot be standardised and requires large experience.

Another method is the terminal deoxynucleotidyl transferase (TdT)-mediated endlabeling of the DNA strand breaks (TUNEL). The TUNEL method detects DNA strand breaks in cells undergoing apoptosis. TdT is an enzyme which catalyzes the addition of deoxyribonucleotide triphosphate to the 3'-OH ends of double or single-stranded DNA. Unlike normal cells, apoptotic cell nuclei incorporate exogenous nucleotides (dUTP)-DIG in the presence of TdT. An anti-DIG antibody fragment with a conjugated fluorochrome enables the visualisation of apoptotic cells. An increase of apoptotic cells causes a higher number of DNA fragments and consequently a brighter fluorescence. An advantage of this method is the very high specificity [21]. A disadvantage of this method is that it is expensive and can only be used for a small set of samples, because it is time intensive. Therefore, it is not applicable for large screening programmes.

The loss of cell membrane polarity and the presentation of increased amounts of phosphatidyl serine (PS) on the outside of the cell membrane during the early phase of apoptosis has led to yet a new approach. Annexin V is a calcium-dependent phospholipid binding protein with high affinity for PS. Due to the fact, that the cell membrane integrity is maintained in the early and intermediate phases of apoptosis but not in necrosis, it is possible to distinguish between apoptotic and necrotic cells, when Annexin V is used concomitantly with the DNA dye PI. Early and intermediate apoptotic cells show increased binding of Annexin-FITC and are mainly negative for PI-staining. Late apoptotic stages and necrotic cells become double positive, because of PS presentation on the surface and the PI staining of intracellular nucleic ac-

ids due to disintegration of the membrane [22]. This method
is also costly and labour intensive.

Green fluorescence protein (GFP) from the
jellyfish *Aequorea victoria* can be used to monitor gene
expression and protein localization in living organisms
(in vivo) and in vitro [23-26]. GFP-fluorescence is stable,
can be monitored noninvasively in living cells and per-
sists in paraformaldehyde-fixed cells. FACS-optimized mu-
tants of green fluorescence protein have been developed
[8]. One of these mutants (GFPmut1) has been integrated
into the pEGFP vectors and is commercially available
(from Clontech). The big advantage of this mutant is that
the maximal excitation peak of GFPmut1 is 488 nm and the
emission maxima is 507 nm. Conventional flow cytometers
are equipped with an argon laser emitting light at 488 nm
and have the suitable detection filters already in-
stalled, making the GFPmut1-protein an ideal candidate
for flow cytometry studies and fluorescence microscopy.

GFP has already been used as marker for visu-
alizing changes in cell morphology such as blebbing
caused by cytotoxic agents or apoptosis, or as transfec-
tion marker (WO97/11094), or as marker for screening fac-
tors modulating gene expression (WO97/14812). GFP has
also been used as a marker protein to detect the progres-
sion of the morphological changes of apoptotic cells[37].

GFP has been used as a marker-protein to de-
tect cells transiently transfected with the commercially
available plasmid pEGFP-C1 (Clontech) [34]. According to
Lamm et al.[34] apoptosis was detected by reduced fluores-
cence of the DNA-binding dye PI in the apoptotic subpopu-
lation. It was not recognized that GFP itself could be
used as a marker for apoptosis. The great disadvantage of
PI-staining is that no changes in the state of one and
the same cells can be monitored but only one specific
state since for PI-staining the cells have to be perme-
abilized and fixed.

Experiments by the present inventors have shown, that stable transfection of eukaryotic cells (e.g. A20.2J) with the pEGFP-C1 (as received from the manufacturer Clontech with the cytomegalovirus CMV promoter) used in the above cited state of the art for tansient transfection results in little or no expression of the GFPmut1 gene.

For the foregoing reason there is a need for a new and improved method and tools for determining apoptosis and necrosis of cells which in particular can be used to efficiently and cheaply assay compounds on their pro-apoptotic or anti-apoptotic or necrotic activity.

Disclosure of the Invention

Accordingly, objects of the invention are to provide an improved method for the determination of apoptotic and/or necrotic conditions of cells and to provide improved tools useful for this method.

The present invention is directed to a method for the determination of apoptotic and/or necrotic conditions of living test cells that satisfies the hereinbefore discussed needs. This method comprises monitoring changes in the signal or the intensity, respectively, of a marker protein in said test cells, in particular a method wherein the apoptotic and/or necrotic condition is monitored in the presence of a non-, pro- or anti-apoptotically or necrotically active compound, and/or a physical stimulus, and wherein the marker protein is preferrentially produced in the test cells after stable transfection of said cells with a DNA coding for and expressing the marker protein.

Preferred marker proteins are fluorescent marker proteins with the Green Fluorescent Protein (GFP) or a fluorescent mutant thereof, e.g. GFPmut1, being particularly preferred.

Another object of the present invention is a
new vector for the transfection, preferably the stable
transfection of test cells which are able to highly ex-
press said marker protein, preferably a fluorescent pro-
5   tein, in particular GFP or a mutant thereof. It was found
that the known hEF-1α promoter, and the new combination
of the CMV and the MoLV-LTR promoter are particularly
well suited for enhancing transcription of the GFPmut1
gene.

10   Another object of the present invention are
live cells or live cell lines, respectively stably trans-
fected with such a vector. Such a cell line can be used
in an assay to determine non-, pro- or anti apoptotic or
necrotic activity of test compounds. Although it is pos-
15 sible to transfect live cells with more than one vector
with different marker molecules, for e.g. the simultane-
ous detection of different signals, usually one marker is
sufficient.

Another object of the present invention is a
20 method to assay the non-, pro or anti-apoptotic or ne-
crotic activity of a test compound on live test cells.
This method comprises stably transfecting a group of said
cells with at least one vector coding for and expressing
a marker protein. The transfected cells are treated in a
25 suitable culture medium with a test compound. The change
in the signal or intensity of a signal, respectively,
i.e. the decrease or increase, of the expressed marker
protein in said group of cells is monitored by conven-
tional methods, and compared with the results observed
30 with a parallel group of the same test cells which was
not exposed to the test compound but otherwise identi-
cally treated. The test compound may consist of a multi-
plicity of compounds, e. g. as obtained from combinatori-
cal chemistry methods. Of particular interest are the
35 apoptotic or necrotic conditions of normal and cancer
cells under the influence of test compounds and/or physi-
cal stimuli.

In a specific embodiment of the methods of the present invention the test cells or cell line are transfected with a test gene expressing a protein of interest such as an apoptosis inhibitor or an apoptosis
5   stimulator.

These and other features, aspects, and advantages of our invention will become better understood with reference to the following description of modes for carrying out the invention, the appended claims and accompa-
10   nying drawings.

Brief Description of Drawings

15   Fig. 1: pBluescriptIIKS(+)+EF-1α plasmid. The promoter of human EF-1α has been cloned into the pBluescriptIIKS (+)vector (from Stratagene) with Hind III and Xba I.

Fig. 2: pBluescriptIIKS(+)+EF-1α +GFP plas-
20   mid. The gene coding for GFPmut1 (from pEGFP-C1, Clontech) has been cloned into the pBluescriptIIKS(+)+EF-1α plasmid with Xba I and Ssp I.

Fig. 3: pEGFP-N1+MoLV-LTR plasmid. The MoLV-LTR promoter has been cloned into the pEGFP-N1 vector
25   (from Clontech) with Hind III and Eco RI.

Fig. 4: FACS-histogramm showing the difference of the fluorescing capacities between non-transfected living A20 and GFP-transfected living A20GFP measured on the FL-1 channel.
30   Fig. 5: FACS-histogramm showing the difference of the fluorescing capacities between non-transfected living PB3c and GFP-transfected living PB3cGFP measured on the FL-1 channel.

Fig. 6a: FACS-histogramm showing the differ-
35   ence of the fluorescing capacities between non-transfected living Jurkat and GFP-transfected living JurkatGFP measured on the FL-1 channel.

Fig. 6b: FACS-histogramm showing the fluorescence intensity of living JurkatGFP compared to apoptotic JurkatGFP (induced by recombinant human TRAIL)

Fig. 7a: FACS dot plot showing the cell size
5 (FSC) and granularity (SSC) of living A20GFP.

Fig. 7b: FACS-histogramm showing the typical GFP-associated fluorescence profile of living A20GFP.

Fig. 8a: FACS dot plot showing the shrinkage and increased granular density (compared to Fig. 7a) of
10 sFasL SN treated and therefore mainly apoptotic A20GFP.

Fig. 8b: FACS-histogramm showing the decrease of GFP associated fluorescence in sFasL SN treated and therefore mainly apoptotic A20GFP.

Fig. 8c: FACS dot plot showing the difference
15 between high fluorescing living A20GFP (R1) and low fluorescing apoptotic A20GFP (R2).

Fig. 8d: FACS dot plot of high fluorescing living A20GFP (gate R1 from Fig. 8c). The high fluorescing A20GFP cells (R1) belong mainly to the not shrunken
20 and not granular dense cell population (typical pattern of living cells).

Fig. 8e: FACS dot plot of low fluorescing apoptotic A20GFP (gate R2 from fig. 8c). The low fluorescing A20GFP cells belong mainly to the shrunken and
25 granular dense cell population (typical feature of apoptotic cells).

Fig. 9a: FACS dot plot showing the cell size and granularity pattern of living A20GFP (ethanol treated and PI-stained).

30 Fig. 9b: FACS-histogramm showing the typical DNA-pattern of PI-stained living A20GFP.

Fig. 10a: FACS dot plot showing the shrinkage of sFasL SN treated A20GFP (ethanol treated and PI-stained).

35 Fig. 10b: FACS-histogramm showing the fragmentation of DNA (represented by the sub-G1 peak = M1), as proof for apoptosis in sFasL SN treated A20GFP.

Fig. 11: FACS-histogramm showing the fluores-
cence intensity of living A20GFP compared to apoptotic
A20GFP, necrotic A20GFP and living non-transfected A20.

Fig. 12a: FACS-histogramm showing the induc-
tion of apoptosis by sFasL SN in A20GFP (control experi-
ment to Fig. 12b).

Fig. 12b: FACS-histogramm showing the induc-
tion of apoptosis by sFasL SN in A20GFP in the presence
of ActD and CHX. Neither a reduction of GFP-associated
fluorescence nor a inhibition of apoptosis can be ob-
served.

Fig. 13: FACS-histogramm showing the anti-
apoptotic effect of IL-3 treatment. Apoptotic PB3cGFP
cells show a lower GFP-associated fluorescence intensity
compared to the IL-3 treated and therefore living
PB3cGFP.

Fig. 14: FACS-histogramm showing the pro-
apoptotic effect of sFasL SN on JurkatGFP, visualized as
a decrease of GFP-associated fluorescence in apoptotic
JurkatGFP cells compared to living JurkatGFP.

Fig. 15: FACS-histogramm showing the differ-
ence of the fluorescing capacities between non-
transfected living DM and GFP-transfected living DMGFP
measured on the FL-1 channel.

Fig. 16: pEGFP-N1+MoLV-LTR plasmid containing
a hygromycin gene (from pREP4, Invitrogen). The MoLV-LTR
promoter (from pLXSN, Clontech) has been cloned into the
pEGFP-N1 vector (from Clontech) with Sma I and Sac II.
The hygromycin gene (from pREP4 digested with Nru I) has
been cloned into the Ase I predigested pEGFP-N1+MoLV-LTR
vector.

Modes for Carrying out the Invention

Apoptotic and/or necrotic conditions of cells
can be induced either physically, e.g. by irradiation, or

chemically, e.g. by treatment with pro-apoptotically or
necrotically or other active compounds.

    Marker proteins, that can be used for the
method, are proteins which are detectable by their physi-
5 cal and/or chemical characteristics, e.g. by fluores-
cence, thereby allowing the measurement of a signal at at
least two different times, i.e. a change in the signal.
Suitable marker proteins furthermore have to change their
signal dependent on the live and apoptotic and/or ne-
10 crotic state of the cells. They can be directly intro-
duced into the cells or produced internally after trans-
fection with a vector expressing the marker protein. Di-
rect introduction of the marker protein may be achieved
by methods known in the art, e.g. by the use of lipo-
15 somes. Test cells are transfected either transiently,
e.g. with a lipid such as the LipofectAMINE ™ reagent, or
preferentially stably by use of a gene-gun or other suit-
able methods, and preferably by electroporation or with
the SuperFect Reagent. Useful vectors are of RNA or pref-
20 erably of DNA origin. The marker protein is produced in
the test cells after transfection with the vector, usu-
ally a DNA vector, coding for and expressing the marker
protein. Useful marker proteins are known and comprise
any detectable proteins, in particular fluorescent pro-
25 teins, e.g. the blue fluorescent protein (BFP), a green
fluorescent protein such as GFPuv or GFPwt. A preferred
marker protein is the GFPmut1.

    The amount of intact marker protein (marker
function) present in the cells can be visualized by the
30 physical and/or chemical characteristics or interactions
of the marker protein, preferably by fluorescence. Under
necrotic conditions the marker protein loses its function
much faster and more completely compared to apoptotic
conditions. Under necrotic conditions the marker function
35 is lost within a few hours (usually 100 % necrosis is ob-
tained within 1 to 2 hours), whereas functional loss is
delayed under apoptotic conditions. Contrary to necrosis,

apoptosis is dependent on the specific cell line and the specific apoptosis inducer, and usually takes 8 hours or more. The difference in time also allows the distinction between these two cell conditions.

5   Useful visualisation is performable on live cells, preferably on live cells in the culture medium either by color reactions or preferentially by fluorescence measurements. Detection devices comprise flow cytometry, e.g. FACScan™, fluorescence platereaders, e.g. FLIPR™,

10 or any other suitable detection device. The acquisition device used is a FACScan™. The following parameters are preferentially measured:

- FSC-Height (forward scatter) as a measure for the cell size,

15   - SSC-Height (side scatter) as a measure for the internal granularity (density) of the cells,

- green fluorescence (e.g. of GFPmut 1) is visualised on the FL-1 channel (FL-1 Height) in the FACScan™.

20   The results are compared after dot plot and/or histogramm visualisation (see Fig. 4 to 15).

Useful test cells comprise eukaryotic and prokaryotic cells. Prokaryotic cells include bacterial

25 and cyanobacterial cells. Eukaryotic cells include mammalian, fungal, insect, avian, worm, fish, crustacean, reptilian, amphibian and plant cells as well as cell lines thereof. The test cells usable in the method are cells of any type, preferentially normal, i.e. genetically

30 cally non-altered, infected, e.g. with virus, parasites, bacteria or prions infected, tumor cells or genetically manipulated or altered cells of human or animal origin, which can be cultured in vitro, carrying a vector expressing the marker gene, but also marker gene trans-

35 formed bacteria that can be used in antibiotica screening. Such test cells are for example lymphoma cells, e.g. A20.2J, Jurkat, mast cells, e.g. PB-3c, or melanoma

cells, e.g. DM, which are transfected with DNA vectors
pBluescriptIIKS(+)+EF-1α +EGFP or pEGFP-N 1 +MoLV-LTR,
respectively, to give the live cell lines named A20GFP,
PB3cGFP, JurkatGFP or DMGFP, respectively.

5   It was found that transcription and transla-
tion modulation, e.g. inhibition, is insignificant to the
method of the present invention.

          For the same reason also direct introduction
of marker protein or expression of marker protein in
10   transiently transfected cells leads to good results.

          However, for studies including reproducibil-
ity studies it is much preferred to use stably trans-
fected cell lines. Stably transformed cell lines that are
highly and stably expressing over long times are obtain-
15   able with the above mentioned DNA vectors pBluescript-
IIKS(+)+EF-1α+EGFP and pEGFP-N1+MoLV-LTR or variants
thereof comprising further selection markers such as a
hygromycin encoding gene (see e.g. Figure 16) and/or a
gene expressing a protein to be examined, e.g. a possible
20   or known apoptosis inhibitor or stimulator.

          With stably transfected cell lines comprising
one of the above mentioned vectors, very reliable and
very good reproducible results were obtained with differ-
ences in the % range detectable.

25   In a preferred method the presence of Green
Fluorescent Protein (GFP) or a fluorescent mutant thereof
(e.g. GFPmut1) is monitored by means of a flow cytometer
measuring the fluorescence intensity. In general two
groups of test cells are used, each of a defined number
30   of cells (e.g. cancer cells), which are transfected with
a DNA vector according to our invention. Depending on the
detection device the number of test cells in the test
tubes is 1 to 50'000'000, usually 5 to 50'000'000, but
for flow cytometry 10'000 to 500'000 are preferably used.
35   In a preferred method two groups of test cells are used,
each of a defined number of cells (e.g. cancer cells),
which are stably transfected with any of the vectors ac-

cording to the invention, incubating one group together with the test compound in a culture medium, stimulating the cells of both groups with an excitation beam, usually with a wave lenght of high energy, in particular an argon

5 laser, determining the fluorescing intensity of the cells of each group by means of a flow cytometer, presently on the FL-1 channel (FL-1 Height), and comparing the obtained fluorescing intensity of the cells of the two groups.

10   Another embodiment of the invention is directed to vectors useful in the method described above. A vector according to the invention comprises a gene coding for the marker protein which is operably linked to one or more strong promoters, preferably the hEF-1α promoter,

15 the MoLV-LTR promoter or a combination of the CMV and the MoLV-LTR promoter. The promoter of EF-1α (elongation factor-1α) chromosomal gene efficiently stimulates in vitro transcription [28]. EF-1α promotes the GTP-dependent binding of an aminoacyl-tRNA to ribosomes in eukaryotic

20 cells. EF-1α is one of the most abundant proteins in eukaryotic cells and is expressed in almost all kinds of mammalian cells. The MoLV-LTR (Moloney Leukemia Virus-Long Terminal Repeat) is a very effective promoter [33]. These findings make the promoter of EF-1α and the MoLV-

25 LTR promoter preferred candidates for enhancing transcription of a desired gene.

        Preferred vectors have a gene coding for the GFP or a fluorescent mutant thereof. They are in particular able to express the marker protein GFPmut1. Such vec-

30 tors are specifically the plasmid pBluescript II KS(+)+EF-1α +EGFP and pEGFP-N1+MoLV-LTR.

        Advantageously vectors with one or more strong promoters are used in order to obtain a high expression of the gene coding for the marker protein, pref-

35 erably GFPmut 1.

        The present vectors are produced by methods known in the art.

Another embodiment of the invention is directed to live cell lines useful in the above method. Preferred living cell lines are transfected with a preferred vector according to the invention and are named
5   A20GFP, PB3cGFP, JurkatGFP or DMGFP.

A20.2J is preferentially transfected with the pBluescriptIIKS(+)+EF-1α +EGFP vector, whereas Jurkat, PB-3c, and DM are transfected with the pEGFP-N 1 +MoLV-LTR vector. Suitable methods to transfect cells are known
10   to the skilled person and are e.g. electroporation or with the SuperFect Reagent. The transfected cells are selected by single cell assay in order to obtain clonally and stably transfected cell lines. The selection process involves high GFP expression (high fluorescence inten-
15   sity) and GFP-fluorescence of the selected cells in a narrow range, in order to obtain a homogenous high GFP-expressing cell line.

Cells or cell lines suitable for the methods of the present invention are also cells or cell lines,
20   transfected with a test gene, i.e. a gene the effect of which on apoptosis or apoptotic conditions shall be studied such as apoptosis inhibitors or stimulators, also called anti-apoptotic or pro-apoptotic genes. Such cells or cell lines are also suitably usable in drug screening
25   methods or to determine the effect of test substances applied to such cells or cell lines.

Anti-apoptotic genes are for example bcl-2, bcl-x_L and FLIP (FLICE inhibitory protein), and pro-apoptotic genes are for example bax, bcl-x_S and bad.
30   Methods and vectors to produce transfected cells are known to the skilled person and are applicable for the production of cells or cell lines, respectively, that are suitable for the present invention and transfected with an anti-apoptotic or pro-apoptotic gene.
35   Whereas for the transfection with GFP the vectors of the present invention are preferred, the transfection with anti-apoptotic or pro-apoptotic genes can be performed

with any vector suitable for, preferably stable, trans-
fection.

In another embodiment the invention is di-
rected to an assay to determine the non-, pro-, or anti-
5   apoptotic or necrotic activity of new or known test com-
pounds. The test is performed in that a defined number of
transfected and selected cells is incubated with a test
compound or/and a physical stimuli, e.g. sonication or
irradiation, in the appropriate culture medium. Preferen-
10   tially 250'000 cells per condition are used. Any culture
medium can be used in which the cells survive and/or
grow. Preferred culture media comprise e.g. Iscove's for
A20GFP and DMGFP, Iscove's plus IL-3 for PB3cGFP, and
RPMI for JurkatGFP.

15   For comparative and standardisation purposes
useful apoptotic compounds are e. g. proteins, such as
FasL, fatty acids, e.g. ceramide, steroids, e.g. corti-
sol, antibiotics, e.g. bleomycin, and others. A preferred
apoptotic substance for A20GFP is FasL. A preferred apop-
20   totic substance for JurkatGFP is FasL or TRAIL. A useful
anti-apoptotic substance for PB3cGFP is IL-3. Toxicity
and thereby necrosis can be induced with a wide variety
of substances. Preferred induction of necrosis includes
the use of antibodies plus complement on A20GFP.

25   Cells can be cultured at different tempera-
tures, but for the assay the test cells are preferably
cultured at 37° during the time of the assay. In FACS-
analysis 10 000 cells are usually evaluated from a pool
of 250 000 cells.

30   Live, transfected cells, in comparison to
apoptotic transfected cells, show a high level of spe-
cific fluorescence intensity, whereas apoptotic cells
lose part of their fluorescent capacity after some hours
(shift to the left on the fluorescence detecting channel
35   FL-1). On the other hand, necrotic cells lose all their
fluorescing capacity within a couple of hours and at that

time render them indistinguishable from non-transfected cells.

The reduction of the experimental set-up to a single parameter (a marker protein, e. g. GFPmut1) and the possibility of standardisation renders the present invention a useful and improved method for the screening of non-, pro-, antiapoptotic and necrotic substances and/or physical stimuli. Standardisation is possible for the transfection and selection method, the fluorescence measuring, the numbers of transfeced cells, the amount of test compounds used, the type and intensity of the physical stimuli, and other standardizable parameters, such as the setup of the flow cytometer and/or any other suitable screening device, e. g. fluorescence platereaders.

Although the present invention has been described in considerable detail with reference to certain preferred embodiments thereof, it should be noted that other embodiments or versions are possible. For example for the detection of the effect of non-, pro- or anti apoptotically or necrotically active compounds on specific cell lines, e.g. specific cancer cell lines, such as cell lines of malignant tumors of human breast, lung, brain, colon, prostate and the like, the relevant cell line may be stably transfected with a vector of the invention, or vectors encoding other easily detectable proteins may be constructed and used for transfection. Therefore the spirit and scope of the appended claims should not be limited to the description of the preferred versions contained therein.

The following examples describe the invention in still more detail but they also should not be construed as a limitation of the present invention.

Example 1: Construction of pBluescriptIIKS(+)+EF-1α+EGFP

        For the construction of the pBluescrip-
tIIKS(+)+EF-1α+EGFP plasmid (see Fig. 2) the promoter of
human EF-1α (from pEF-BOS, kindly provided by S. Nagata
[27]) and the gene EGFP + SV40 poly A coding for GFPmut1
5 (from pEGFP-C1, Clontech) were cloned into the
pBluescriptIIKS(+) vector (from Stratagene).

        The first step was to clone the promoter of
human EF-1α (from pEF-BOS) into the Xba I and Hind III
predigested pBluescriptIIKS(+) vector.

10   The resulting EF-1α promoter fragment, after
Hind III and Xba I digestion of pEF-BOS, has a length of
1188 bp, whereas the other resulting fragment corresponds
to the pEF-BOS backbone.

        The pBluescriptIIKS(+) contains a unique Xba
15 I restriction site (position 677) and a unique Hind III
restriction site (position 719). The two resulting frag-
ments after Xba I and Hind III digestion have a length of
2919 bp and 42 bp, respectively.

        Three µg pEF-BOS vector were mixed in an Ep-
20 pendorf tube (tube a) with 1 µl Hind III (from Boehringer
Mannheim, 12 U/µl), 1 µl Xba I (from New England Biolabs,
20000 U/ml), 3 µl 10x incubation buffer for restriction
enzyme B (from Boehringer Mannheim) and 20 µl double dis-
tilled water making a final volume of 30 µl. In another
25 Eppendorf tube (tube b), 3 µg pBluescript II KS(+) were
pipetted together with 1 µl Hind III, 1 µl Xba I, 3 µl
10x incubation buffer for restriction enzyme B and 20 µl
double distilled water to make a final volume of 30 µl.
Both Eppendorf tubes (tubes a and b) were incubated for 3
30 hours at 37°C. After incubation, 11 µl DNA-loading buffer
(0.25% bromophenol blue from Sigma, 0.25% xylene cyanol
from Sigma and 15% Ficoll type 400 from Pharmacia in wa-
ter) were added to both tubes and thereafter the contents
were loaded onto separate slots on a 0.6% low-melting-
35 point (LMP) agarose (from Bethesda Research Laboratories)
gel. In a third slot, 9 µl 1 Kb DNA-ladder (from Pro-
mega), diluted 1:3 with DNA-loading buffer, were loaded

on the gel. The 0.6% LMP agarose gel is prepared by mix-
ing 0.3 g LMP-agarose in 50 ml 1x TAE (50x TAE concen-
trated stock solution (per liter): 242 g
Tris(hydroxymethyl)-aminomethane (from Merck), 57.1 ml
5   glacial acetic acid (from Merck), 100 ml 0.5 M EDTA (from
Sigma) (pH 8.0) and double distilled water to a final
volume of 1 liter), heating the mixture until it is dis-
solved and adding ethidiumbromide solution (from Sigma)
to a final concentration of 1 µg/ml. The gel is run at
10   60-70 Volts for 60-90 minutes. The ethidiumbromide
stained DNA-bands in the gel are visualized with 300 nm
UV-light and their size are determined relative to the 1
Kb DNA-ladder. The DNA-band containing the 1188 bp long
fragment (EF-1α promoter) from the digested pEF-BOS vec-
15   tor and the DNA-band containing the 2919 bp long fragment
from the digested pBluescript II KS(+) were cut out from
the LMP agarose gel and put into two different Eppendorf
tubes. Both tubes were centrifuged briefly and incubated
at 65 °C for 5 minutes. Afterwards, 3.5 µl of the 2919 bp
20   long fragment (Hind III and Xba I digested pBluescript II
KS(+)) were mixed together with 7 µl of the 1188 bp long
fragment (EF-1α promoter from the Hind III and Xba I di-
gested pEF-BOS) in a separate Eppendorf tube, incubated
at 65 °C for 5 minutes and centrifuged briefly. The mix-
25   ture was cooled at 37 °C and then the following ligation
mix was added: 2 µl 10x ligation buffer (from Appligene,
provided with T4 DNA ligase), 7.5 µl double distilled wa-
ter and 1 µl T4 DNA ligase (from Appligene, 5 U/µl). The
ligation reaction was incubated overnight at 15 °C.
30   The newly ligated pBluescriptIIKS(+)+EF-1α
was transformed into One Shot™ competent _E. coli_ (from
Invitrogen), according to the manufacturer protocol of
Invitrogen.

           10 µl of the ligation reaction were used for
35   the transformation of competent _E. coli_. 50, 100 and 200
µl of the transformation mixture were plated on different
ampicillin-containing LB agar plates (10 g tryptone, 5 g

yeast extract, 10 g NaCl, 20 g bactoagar (from Difco La-
boratoires Detroit Michigan USA), adjust pH to 7.0 with
NaOH, water to a final volume of 1 liter, autoclave, cool
the agar-media to 55 °C, add ampicillin solution to a fi-
5   nal concentration of 50 µg/ml and pour the plate with 10-
15 ml agar/plate). The plates/transformation mix were
then incubated at 37 °C overnight. The next day different
bacterial colonies were picked for further analysis of
recombinant DNA clones by small-scale plasmid DNA isola-
10 tion (according to the alkaline lysis method described in
Maniatis et al. 32).

To check the presence of the EF-1α promoter
fragment cloned into pBluescript II KS(+), a Hind III and
Xba I restriction enzyme digestion of the isolated DNA
15 was carried out (3 µl 10x incubation buffer for restric-
tion enzyme B, 5 µl plasmid DNA from the alkaline lysis
method, 22 µl double distilled water, 1 µl Hind III, 1 µl
Xba I were mixed together in an Eppendorf tube and incu-
bated for 3 hours at 37 °C), loaded on a 1% agarose (from
20 Bio-Rad) gel and subsequently visualized with 300 nm UV-
light and analyzed. The expected fragment of 1188 bp
length after Hind III and Xba I digestion was observed,
and therefore confirmed the presence of the cloned frag-
ment (see also Fig. 1).

25   In order to obtain large quantities of the
newly created plasmid, a large-scale plasmid DNA isola-
tion has been performed according to the Qiagen Plasmid
Maxi Protocol.

The second step was to clone the EGFP + SV40
30 poly A (from pEGFP-C1) into the Xba I and Ssp I predi-
gested pBluescript II KS(+) + EF-1α.

The pEGFP-C1 vector (from Clontech) contains
a unique Nhe I restriction site (position 592) and two
Ssp I restriction sites (position 1664 and 2217). The
35 three resulting fragments, after Nhe I and Ssp I diges-
tion, have a length of 3106 bp, 1072 bp and 553 bp, re-
spectively.

The pBluescriptIIKS(+)+EF-1α (see Fig. 1) contains an Xba I and two Ssp I restriction sites and the three resulting fragments, after Xba I and Ssp I digestion, have a length of 3319 bp, 658 bp and 130 bp, re
5 spectively.

Of the pEGFP-C1 vector 4.2 µg were mixed in an Eppendorf tube (tube 1) with 1 µl Nhe I (from New England Biolabs, 4000 U/ml), 1 µl Ssp I (from Boehringer Mannheim, 10 U/µl), 3 µl 10x incubation buffer for re
10 striction enzyme M (from Boehringer Mannheim) and 18 µl double distilled water making a final volume of 30 µl. In another Eppendorf tube (tube 2), 3-4 µg pBluescriptIIKS(+)+EF-1α were pipetted together with 1 µl Xba I, 1 µl Ssp I, 3 µl 10x incubation buffer for restriction en
15 zyme H (from Boehringer Mannheim) and 18 µl double distilled water to make a final volume of 30 µl. Both Eppendorf tubes (tubes 1 and 2) were incubated for 4 hours at 37°C. After incubation, 11 µl DNA-loading buffer were added to both tubes and thereafter the contents were
20 loaded into separate slots on a 0.6% low-melting-point (LMP) agarose gel. In a third slot, 9 µl 1 Kb DNA-ladder, diluted 1:3 with DNA-loading buffer, were loaded on the gel. Run the gel at 60-70 Volts for 60-90 minutes. The ethidiumbromide stained DNA-bands in the gel are visual
25 ized with 300 nm UV-light and their sizes are determined relative to the 1 Kb DNA-ladder. The DNA-band containing the 1072 bp long fragment from the digested pEGFP-C1 vector and the DNA-band containing the 3319 bp long fragment from the digested pBluescript II KS(+)+EF-1α were cut out
30 from the LMP agarose gel and put into two different Eppendorf tubes. Both tubes were centrifuged briefly and incubated at 65 °C for 5 minutes. Afterwards, 3.5 µl of the 3319 bp long fragment (Xba I and Ssp I digested pBluescript II KS(+) + EF-1α) were mixed together with 7
35 µl of the 1072 bp long fragment (EGFP + SV40 poly A from the Nhe I and Ssp I digested pEGFP-C1) in a separate Eppendorf tube, incubated at 65 °C for 5 minutes and cen-

trifuged briefly. The mixture was cooled at 37 °C and
then the following ligation mix was added: 2 μl 10x liga-
tion buffer, 7.5 μl double distilled water and 1 μl T4
DNA ligase. The ligation reaction was incubated overnight
5   at 15 °C.

The newly ligated pBluescriptIIKS(+)+EF-
1α+EGFP was transformed into One Shot™ competent E. coli
(from Invitrogen), according to the manufacturer protocol
of Invitrogen. Ten μl of the ligation reaction were used
10   for the transformation of competent E. coli. 50, 100 and
200 μl of the transformation mixture were plated on dif-
ferent ampicillin-containing LB agar plates and incubated
at 37 °C overnight. The next day different bacterial
colonies were picked for further analysis of recombinant
15   DNA clones by small-scale plasmid DNA isolation (accord-
ing to the alkaline lysis method described in Maniatis et
al. [7]).

The newly constructed pBluescript IIKS(+)+EF-
1α+EGFP vector (see Fig. 2) contains two Sal I restric-
20   tion sites, one in the pBluescriptIIKS(+) backbone and
the other one in the cloned EGFP+SV40 poly A fragment.
The two resulting fragments after a Sal I digestion have
a length of 1981 bp and 2410 bp, respectively.

In order to check the presence of the EGFP +
25   SV40 poly A fragment cloned into pBluescriptIIKS(+)+EF-
1α, a Sal I restriction enzyme digestion of the isolated
DNA was carried out (3 μl 10x incubation buffer for re-
striction enzyme H, 5 μl plasmid DNA from the alkaline
lysis method, 22 μl double distilled water, 1 μl Sal I
30   (from Appligene, 10 U/μl) were mixed together in an Ep-
pendorf tube and incubated for 3 hours at 37 °C), loaded
on a 1% agarose gel and subsequently visualized with 300
nm UV-light and analyzed. The two expected fragments of
1981 bp and 2410 bp length after Sal I digestion were ob-
35   served, and therefore confirmed the presence of the
cloned fragment.

In order to obtain large quantities of the
newly created plasmid (see Fig. 2), a large-scale plasmid
DNA isolation has been performed according to the Qiagen
Plasmid Maxi Protocol.

5   At this point, to check the functionality of
the newly constructed pBluescriptIIKS(+)+EF-1α+EGFP plas-
mid, COS-1 cells (COS-1 is a fibroblast-like cell line
established from CV-1 simian cells (ATCC CCL 70), from
ATCC CRL 1650) were transiently transfected with 2 μg of

10   the plasmid, according to the DEAE-Dextran Transfection
Protocol from the Mammalian Transfection Kit of Strata-
gene. After 1-3 days incubation at 37 °C, the transiently
transfected COS-1 cells were analyzed under the fluores-
cence microscope and highly fluorescing GFP-positive COS-

15   1 cells have been observed, indicating that the con-
structed GFP-carrying plasmid was functional.

Example 2: Construction of pEGFP-N1+MoLV-LTR

20   For the construction of the pEGFP-N1+MoLV-LTR
plasmid (see Fig. 3) the MoLV-LTR promoter [33] (from Gem-
MoLV-LTR) was cloned into the Hind III and Eco RI predi-
gested pEGFP-N1 vector (from Clontech).

The MoLV-LTR promoter [33] was cloned with Hind
25   III and Eco RI into the pGEM-3Zf(+) vector (from Pro-
mega). The Gem-MoLV-LTR contains a unique Hind III and a
unique Eco RI restriction site. The resulting MoLV-LTR
promoter fragment, after Eco RI and Hind III digestion,
has a length of 697 bp, whereas the other resulting frag-

30   ment corresponds to the pGEM-3Zf(+) backbone.

The pEGFP-N1 (from Clontech) contains a
unique Hind III restriction site (position 623) and a
unique Eco RI restriction site (position 630). The two
resulting fragments, after Hind III and Eco RI digestion,

35   have a length of 4726 bp and 7 bp, respectively.

Of pEGFP-N1 7.3 μg were pipetted in an Eppen-
dorf tube (tube A) together with 1 μl Hind III, 3 μl 10x

incubation buffer for restriction enzyme B (from Boeh-
ringer Mannheim) and 21 µl double distilled water to
make a final volume of 30 µl. The tube was incubated for
3 hours at 37 °C. Afterwards, it was incubated at 65 °C

5   for 5 minutes, cooled down at 37 °C and 9 µl double dis-
tilled water, 1 µl 10x incubation buffer for restriction
enzyme B and 1 µl Eco RI were added to the mixture making
a final volume of 40 µl. In another Eppendorf tube (tube
B), 3 µg Gem-MoLV-LTR were mixed with 1 µl Hind III, 1 µl

10 Eco RI (from Boehringer Mannheim, 10 U/µl), 3 µl 10x in-
cubation buffer for restriction enzyme B and 20 µl double
distilled water making a final volume of 30 µl. Both Ep-
pendorf tubes (tubes A and B) were incubated for 3 hours
at 37 °C. After incubation, 14 µl DNA-loading buffer were

15 added to both tubes and thereafter the contents were
loaded into separate slots on a 0.6% low-melting-point
(LMP) agarose gel. In a third slot, 15 µl 1 Kb DNA-ladder
(from Promega), diluted 1:3 with DNA-loading buffer, were
loaded on the gel. The gel was run at 60-70 Volts for 60-

20 90 minutes. The ethidiumbromide stained DNA-bands in the
gel were visualized with 300 nm UV-light and their sizes
were determined relative to the 1 Kb DNA-ladder. The DNA-
band containing the 697 bp long fragment from the di-
gested Gem-MoLV-LTR vector and the DNA-band containing

25 the 4726 bp long fragment from the digested pEGFP-N1 were
cut out from the LMP agarose gel and put into two differ-
ent Eppendorf tubes. Both tubes were centrifuged briefly
and incubated at 65 °C for 5 minutes. Afterwards, 3.5 µl
of the 4726 bp long fragment (Hind III and Eco RI di-

30 gested pEGFP-N1) were mixed together with 7 µl of the 697
bp long fragment (MoLV-LTR promoter from the Hind III and
Eco RI digested Gem-MoLV-LTR) in a separate Eppendorf
tube, incubated at 65 °C for 5 minutes and centrifuged
briefly. The mixture was cooled at 37 °C and then the

35 following ligation mix was added: 2 µl 10x ligation
buffer, 7.5 µl double distilled water and 1 µl T4 DNA li-

gase. The ligation reaction was incubated overnight at 15 °C.

The newly ligated pEGFP-N1+MoLV-LTR was transformed into One Shot™ competent *E. coli* (from Invitrogen), according to the manufacturer protocol of Invitrogen.

10 μl of the ligation reaction were used for the transformation of competent *E. coli*. 50, 100 and 200 μl of the transformation mixture were plated on different kanamycin-containing LB agar plates (final concentration of kanamycin: 50 μg/ml) and the plates were then incubated at 37 °C overnight. The next day different bacterial colonies were picked for further analysis of recombinant DNA clones by small-scale plasmid DNA isolation (according to the alkaline lysis method described in Maniatis *et al.* [7]).

To check the presence of the MoLV-LTR promoter fragment cloned into pEGFP-N1, a Hind III and Eco RI restriction enzyme digestion of the isolated DNA was carried out (3 μl 10x incubation buffer for restriction enzyme B, 5 μl plasmid DNA from the alkaline lysis method, 22 μl double distilled water, 1 μl Hind III, 1 μl Eco RI were mixed together in an Eppendorf tube and incubated for 3 hours at 37 °C), loaded on a 1% agarose gel and subsequently visualized with 300 nm UV-light and analyzed. The expected fragment of 697 bp length after Hind III and Eco RI digestion was observed, and therefore confirmed the presence of the cloned fragment (see Fig. 3).

In order to obtain large quantities of the newly created plasmid, a large-scale plasmid DNA isolation has been performed according to the Qiagen Plasmid Maxi Protocol.

Essentially the same method was used for the construction of the pEGFP-N1+MoLV-LTR vector containing a hygromycin gene (Fig. 16), with the following changes. First, the MoLV-LTR promoter (from pLXSN, Clontech) was cloned into the pEGFP-N1 vector with Sma I and Sac II.

Second, the hygromycin gene including a TK promotor and a
TK poly A (from pREP4 (Invitrogen) digested with Nru I)
was cloned into the Ase I predigested pEGFP-N1+MoLV-LTR
vector.

5

      Example 3: Preparation of the cell line ·
A20GFP by electroporation of the murine cell line A20.2J
with pBluescriptIIKS(+)+EF-1α+EGFP

10

      The A20.2J cell line (A20.2J is a BALB/c B
cell lymphoma [29-30] from ATCC TIB 208, American Type Cul-
ture Collection) was co-electroporated with the
pBluescriptIIKS(+)+EF-1α+EGFP and pSV2neo (plasmid con-
15 taining the neo gene which confers resistance to the drug
G418). 3×10^7 cells (A20.2J) were required for the elec-
troporation and were plated into fresh medium complete
(Iscove's modified Dulbecco's medium (from Gibco BRL)
supplemented with 5 % fetal calf serum (from Gibco BRL),
20 0.05 mM 2-mercaptoethanol (from Gibco BRL) and 2 mM L-
glutamine (from Gibco BRL)) one day prior to the pora-
tion. Before electroporation, the cells were washed three
times in ice-cold Ca^{2+}/Mg^{2+} free PBS (phosphate buffered
saline: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g
25 KH_2PO_4 in 800 ml double distilled water, adjusted to pH
7.4 with HCl and double distilled water was added to a
final volume of 1 liter), counted and 3×10^7 cells were
resuspended in 800 μl ice-cold PBS. 60 μg pBluescript-
IIKS(+)+EF-1α+EGFP and 6 μg pSV2neo were aliquoted into
30 an electroporation cuvette for the co-electroporation of
A20.2J. The chilled cells were added to the DNA solution
in the electroporation cuvette. The cells and the DNA so-
lution were mixed by pipetting and incubated on ice for
15 minutes. After incubation, the DNA cell suspension was
35 subjected to an electric pulse of 960 μF and 300 V deliv-
ered by a BioRad Gene Pulser[TM]. After electroporation,
the cells were kept on ice for 15 minutes, followed by a

15 minutes incubation at room temperature. The electropo-
rated A20.2J were then transferred to culture medium (me-
dium complete) and grown for 24 hours before transfec-
tants were selected in medium complete supplemented with
5   G418 (1 mg/ml, from Gibco BRL).

        The electroporated cells were cultured in the
selection medium (medium complete supplemented with G418)
for 2-4 weeks, analyzed under the fluorescence microscope
and then a single-cell-assay was performed in order to
10 obtain a clonally and stably GFP-transfected cell line
named A20GFP.

Example 4: Preparation of the cell line
PB3cGFP by electroporation of the murine cell line PB-3c
with pEGFP-N1+MoLV-LTR

5   The PB-3c cell line (which is a cloned,
strictly IL-3 dependent, non tumorigenic mastocyte line
isolated from the bone marrow of a DBA/2 mouse [31]) was
electroporated with pEGFP-N1+MoLV-LTR. 3×10^7 cells (PB-
3c) were required for the electroporation and were plated
10   into fresh medium (medium complete supplemented with 2 %
IL-3) one day prior to the poration. Before electropora-
tion, the cells were washed three times in ice-cold
Ca^{2+}/Mg^{2+} free PBS, counted and 3×10^7 cells were resus-
pended in 800 µl ice-cold PBS. 10 µg pEGFP-N1+MoLV-LTR
15   were aliquoted into an electroporation cuvette for the
electroporation of PB-3c. The chilled cells were added to
the DNA solution in the electroporation cuvette. The
cells and the DNA solution were mixed by pipetting and
incubated on ice for 15 minutes. After incubation, the
20   DNA cell suspension was subjected to an electric pulse of
960 µF and 300 V delivered by a BioRad Gene Pulser™. Af-
ter electroporation, the cells were kept on ice for 15
minutes, followed by a 15 minutes incubation at room tem-
perature. The electroporated PB-3c cells were then trans-
25   ferred to culture medium (medium complete supplemented
with 2 % IL-3) and grown for 48 hours before transfec-
tants were selected in medium complete supplemented with
2 % IL-3 and G418 (1 mg/ml, from Gibco BRL).

         The electroporated cells were cultured in the
30   selection medium for 2-4 weeks, analyzed under the fluo-
rescence microscope and then a single-cell-assay was per-
formed in order to obtain a clonally and stably GFP-
transfected cell line named PB3cGFP.

35

Example 5: Preparation of the cell line
JurkatGFP by electroporation of the human cell line
Jurkat with pEGFP-N1+MoLV-LTR

5   The Jurkat cell line (Jurkat is a human acute
T cell leukemia from ATCC TIB 152) was electroporated
with pEGFP-N1+MoLV-LTR. 3×10^7 cells (Jurkat) were re-
quired for the electroporation and were plated into fresh
RPMI medium (RPMI 1640 medium (from Gibco BRL) supple-
10   mented with 5 % fetal calf serum (from Gibco BRL), 0.05
mM 2-mercaptoethanol (from Gibco BRL) and 2 mM L-
glutamine (from Gibco BRL)) one day prior to the pora-
tion. Before electroporation, the cells were washed three
times in ice-cold Ca^{2+}/Mg^{2+} free PBS, counted and 3×10^7
15   cells were resuspended in 800 µl ice-cold PBS. 10 µg
pEGFP-N1+MoLV-LTR were aliquoted into an electroporation
cuvette for the electroporation of Jurkat. The chilled
cells were added to the DNA solution in the electropora-
tion cuvette. The cells and the DNA solution were mixed
20   by pipetting and incubated on ice for 15 minutes. After
incubation, the DNA cell suspension was subjected to an
electric pulse of 960 µF and 300 V delivered by a BioRad
Gene Pulser™. After electroporation, the cells were kept
on ice for 15 minutes, followed by a 15 minutes incuba-
25   tion at room temperature. The electroporated Jurkat cells
were then transferred to culture medium (RPMI medium) and
grown for 48 hours before transfectants were selected in
RPMI medium supplemented with G418 (1 mg/ml, from Gibco
BRL).

30   The electroporated cells were cultured in the
selection medium (RPMI medium supplemented with G418) for
2-4 weeks, analyzed under the fluorescence microscope and
then a single-cell-assay was performed in order to obtain
a clonally and stably GFP-transfected cell line named
35   JurkatGFP.

Example 6: Comparison of the fluorescent capacities of non-transfected and GFP-transfected cells

5 Four non-related cell lines (A20.2J, PB-3c, Jurkat and DM) were chosen in order to study the fluorescing capacities.

After stable transfection of the four different cell lines with a GFP-carrying vector, the non-transfected cells were compared with the GFP-transfected
10 cells of each cell line on behalf of their fluorescing capacities, measured by flow cytometric analysis in a FACScan (Becton Dickinson, San Jose, USA). The software programm Cell Quest (Becton Dickinson, USA) was used to acquire the data. The FACScan was calibrated with Cali-
15 brite beads from Becton Dickinson prior to acquisition. The following parameters were measured:

                - FSC-Height (forward scatter) as a measure
for the cell size
20   - SSC-Height (side scatter) as a measure for
the internal granularity (density)of the cells,
                - green fluorescence (eg. GFPmut1) was visu-
alised on the FL-1 channel in the FACScan
                - Orange fluorescence (eg. propidium iodide)
25 was visualised on the FL-2 channel in the FACScan

For measurements, 10000 cells were acquired from each FACS-tube containing 250000 cells.
There were two surprising findings: The first
30 was the high fluorescence intensity of the GFP-transfected cells compared to the autofluorescence of the non-transfected ones (Fig. 4-6 and 15). The second was that the fluorescence-range (visualized on the FL-1 channnel in the FACScan) of all tested live GFP-
35 transfected cell lines was narrow (see Fig.): A20GFP (Fig. 4), PB3cGFP (Fig. 5), JurkatGFP (Fig. 6) and DMGFP

(Fig.15). This rules out the problem of high and low GFP expressors.

5   ## Example 7: Apoptosis assay: induction of apoptosis in A20GFP by sFasL

Apoptosis was induced by an 8 hours incuba-
tion of A20GFP with the sFasL supernatant (sFasL SN has
10   been produced by FasL-transfected N2a cells, which were
provided by A. Fontana, University Hospital, Zurich,
Switzerland). As negative control, A20GFP incubated in
medium complete has been used.

After washing A20GFP cells with PBS, two
15   FACS-tubes (from Falcon) were prepared, each containing
250'000 cells. The cells in the first tube (tube 1) were
resuspended in 500 µl normal medium complete as negative
control, whereas the cells in the second tube (tube 2)
were resuspended in 100 µl apoptosis inducing soluble
20   FasLigand supernatant and 400 µl medium complete to a fi-
nal volume of 500 µl. Both tubes were incubated for 8
hours at 37 °C. After incubation, the cells were washed
with PBS, centrifuged at 1400 rpm for 5 minutes, taken up
in 500 µl PBS and measured in the FACScan.
25   In the control tube (tube 1), the majority of
the cells showed a high GFP-associated fluorescence (Fig.
7a and 7b), whereas in the tube containing the sFasL SN
treated cells (tube 2) a majority of low fluorescing
cells was found after 8 hours incubation (Fig. 8a and
30   8b). These low fluorescing cells corresponded to the
shrunken apoptotic cell population (Fig. 8c and 8e), in
contrast to the high fluorescing cells, that are mainly
found in the bigger and less granular living cell popula-
tion (Fig. 8c and 8d).
35   To further show and confirm, that the low
fluorescing cell population corresponds to the apoptotic
shrunken cell population, a propidium iodide staining for

intracellular DNA has been performed with the remaining
cells of the previously measured tubes (tube 1 and 2).
The cells were first washed with PBS, then incubated in
70 % ethanol at -20 °C for 2 hours, washed again with PBS

5 and stained with 500 µl PI-solution (50 µg PI in 1 ml
PBS) for 30 minutes at 37°C. Both tubes (tube 1 and 2),
containing PI stained cells, were immediately measured in
the FACScan.

        The DNA profile of the apoptosis inducing

10 sFasL SN treated cells (tube 2) was considerably changed
after 8 hours incubation (Fig. 10a and 10b). The majority
of the DNA has been fragmented (visualized as sub-G1 peak
on the FL-2 channel in a FACScan, Fig. 10b), in compari-
son to the control (tube 1, see Fig. 9a and 9b), confirm-

15 ing the result of massive apoptosis induced by sFasL SN.
        Furthermore, the cell population with frag-
mented DNA corresponded to the group of the shrunken
apoptotic and low fluorescing cells.
        In conclusion, apoptotic cells shrink, they

20 have mainly (but not completely) lost the GFP-associated
fluorescence and have a fragmented DNA (which is a spe-
cific characteristic of apoptosis). Moreover, the amount
of apoptotic cells seen with the PI method was comparable
to the amount detected with the GFP method (data not

25 shown).

        Example 8: Toxicity assay: Induction of ne-
crosis in A20GFP

30

        A20GFP cells were first washed with PBS and
then 250000 cells incubated in a FACS-tube with 100 µl
anti-A20 polyclonal antibody for 60 min at 4°C. After in-
cubation, the cells were washed with PBS, resuspended in

35 50 µl rabbit complement (Readysystem) and incubated in a
37 °C watherbath for 1 hour. The cells were washed again
with PBS and resuspended in 500 µl PBS and immediately

measured in a FACScan. As controls, untreated (without
complement) A20GFP and A20 cells were used.

Complement binding induces necrosis in target
cells. This assay was thus performed in order to evaluate
5 the fluorescing capacity of necrotic cells. The findings
were striking, as necrotic cells completely lost their
GFP-associated fluorescence capacity within an hour. Thus
complement treated A20GFP cells can hardly be distin-
guished from non-transfected live A20 cells (Fig. 11).
10 The necrotic cells can be distinguished from apoptotic
cells, the latter still express low fluorescing capacity
(e.g. 8 hours after sFasL SN treatment, as shown in the
example described before). This makes the GFP-assay a
useful tool to distinguish between necrotic, apoptotic
15 and live GFP-transfected cells (Fig. 11).

Example 9: Inhibition of de novo RNA synthe-
sis by actinomycin D (ActD), protein synthesis by cyclo-
20 heximide (CHX) and the potential role of interfering with
the GFP apoptosis assay in A20GFP

To find out whether the fluorescence of
A20GFP and the GFP apoptosis assay as described in exam-
25 ple 7 is dependent on the de novo transcription and pro-
tein synthesis, Act D (pharmacy of the University Hospi-
tal, Basel), a RNA synthesis inhibitor, and Cycloheximide
(Sigma), a protein synthesis inhibitor, were tested in
our experimental protocol. After washing A20GFP cells
30 with PBS, 250000 cells were aliquoted in FACS-tubes and
incubated with:

a) Tube 1 (control): 500 μl medium complete
(MC)

35

b) Tube 2: 100 μl sFasL SN and 400 μl MC
c) Tube 3: 500 μl MC and 5 μg/ml ActD

d) Tube 4: 400 µl MC, 100 µl sFasL SN, 5 µg/ml ActD

e) Tube 5: 500 µl MC and 10 µg/ml CHX

f) Tube 6: 400 µl MC, 100 µl sFasL SN and 10 µg/ml CHX

g) Tube 7: 500 µl MC, 5 µg/ml Act D and 10 µg/ml CHX

h) Tube 8: 400 µl MC, 100 µl sFasL SN, 5 µg/ml Act D and 10 µg/ml CHX

Tubes 1-8 (a-h) were incubated for 10 hours at 37 °C. The fluorescing status of the cells was assayed at 10 hours post treatment.

As seen in figures 12a and 12b, there is no difference between the fluorescence intensity of un-treated A20GFP (Fig. 12a) compared to A20GFP treated with ActD and/or CHX (Fig. 12b). sFasL SN treatment of A20GFP induces apoptosis to the same extent, whether the cells were ActD- and/or CHX-treated or not. In conclusion, ActD and CHX, either alone or together, do not substantially interfere with the fluorescing capacity of A20GFP nor do they inhibit sFasL SN induced apoptosis after 10 hours of treatment. Therefore, A20GFP, in order to fluorescence, do not require de novo transcription and translation dur-ing the 10 hours.

Example 10: Apoptosis assay: induction of apoptosis in PB3cGFP by Interleukin-3 deprivation

PB3cGFP is an interleukin-3 (IL-3) dependent cell line. The addition of IL-3 to the culture medium (medium complete) prevents the PB3cGFP cells from under-going apoptosis (IL-3 as an anti-apoptotic compound). In contrast, upon IL-3 deprivation a strong apoptotic re-sponse is seen within 12 hours.

For this experiment, 250000 PBS-washed PB3cGFP cells were aliquoted in two different FACS-tubes.

Tube A: contained the cells and MC supplemented with IL-3.

5   Tube B: contained the cells and MC without IL-3.

Both tubes were incubated for 12 hours at 37°C.

The GFP-associated fluorescence of the
10 PB3cGFP cells was measured after 12 hours of IL-3 deprivation.

As expected, and seen with A20GFP, very similar results were obtained with PB3cGFP. PB3cGFP, cultured in MC supplemented with IL-3 (tube A), showed a high GFP-
15 associated fluorescence, whereas IL-3 deprived PB3cGFP cells (tube B) underwent apoptosis and demonstrated a low GFP-associated fluorescence intensity, making them clearly distinguishable from live PB3cGFP and live PB3c cells (Fig. 13). These findings have been confirmed by
20 the PI staining method, as it has been described with the A20GFP cells (data not shown).

Example 11: Apoptosis assay: induction of
25 apoptosis in JurkatGFP by sFasL SN

This apoptosis assay has also been performed with sFasL SN, a known cell death inducer in Jurkat cells. 250000 PBS-washed JurkatGFP cells were aliquoted
30 in two different FACS-tubes.

Tube 1 (control): cells were incubated with 500 µl RPMI medium

Tube 2: cells were incubated with 100 µl sFasL SN and 400 µl RPMI medium

35

Both tubes were incubated for 18 hours at 37 °C. In this experiment cells were immediately measured after incubation, without prior washing with PBS.

As expected, and seen with A20GFP and PB3cGFP

5   cells, very similar results were obtained.

As shown in Fig. 14, sFasL SN induced apoptosis. GFP-associated fluorescence was high in live JurkatGFP, whereas the apoptotic JurkatGFP cells had a marked reduced GFP-associated fluorescence, but were

10 still distinguishable from non-transfected live Jurkat cells. These findings have been confirmed by the PI staining method.

In relation to the previous experiment, the present results show that it is not necessary to wash the

15 cells with PBS before measuring. This may be particularly useful for large screening assays, where individual tubes cannot be separately washed.

20   Example 12: Apoptosis assay: Induction of apoptosis in JurkatGFP by recombinant human TRAIL

This apoptosis assay has been performed with recombinant human TRAIL, a known cell death inducer in

25 Jurkat cells. 250'000 JurkatGFP cells were aliquoted in two different FACS-tubes.

Tube 1: (control): cells were incubated with 500 µl RPMI medium,

Tube 2: cells were incubated with 25 µl re-

30 combinant human TRAIL and 475 µl RPMI medium.

Both tubes were incubated for 18 hours at 37° C. After incubation the test tubes were immediately measured without prior washing.

Like sFasL SN, recombinant human TRAIL in-

35 duced apoptosis in JurkatGFP cells (Fig. 6b) in a dose dependent manner (data not shown). Figure 6b shows a similar profile as already observed with A20GFP (Fig. 11)

and PB3cGFP (Fig. 13) cells after induction of apoptosis by sFasL SN or IL-3 deprivation, respectively.

5   Example 13: Preparation of cell line DMGFP by transfection of the human melanoma cell line DM with pEGFP-N1+MoLV-LTR

The human melanoma cell line DM [35] was trans-
10   fected with pEGFP-N1+MoLV-LTR.

10^6 DM cells were plated out one day prior to the transfection in a 10 cm diameter culture dish con-taining complete medium including 10% FCS. 10 µg pEGFP-N1+MoLV-LTR were mixed together with 20 µl SuperFect Rea-
15   gent (Qiagen AG, Switzerland) and 120 µl complete medium w/o FCS and incubated for 30 minutes at room temperature. After incubation, 1 ml complete medium containing FCS were added and the whole mixture was poured onto the dish already containing DM cells in 10 ml complete medium. 48
20   hours after transfection, cells were washed once with complete medium and further cultivated in selective me-dium containing 1mg/ml G418 (Gibco BRL) for 3 weeks until single clones became visible. These clones were analyzed under the fluorescence microscope. Positive clones were
25   further cultivated in 24 well plates in selective medium and analyzed again for the expression of the transgene. Single-cell assay was then performed in order to obtain single clones stably expressing the GFP transgene.

30

Abbreviations

The following abbreviations are used through-out the description:

5

ActD	Actinomycin D
bp	base pairs
CHX	Cycloheximide
DIG	Digoxygenin
ds DNA	double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EF-1α	elongation factor-1α
FACS	Fluorescence Activated Cell Sorter
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
FL-1	Fluorescence channel 1 (detects fluorescence emission in the range of 530 (+/-25) nm
FSC	Forward Scatter (cell size)
GFP	Green Fluorescent Protein
IL-3	Interleukin-3
LMP	low melting point
MC	medium complete
PBS	phosphate buffered saline
PI	propidium iodide
sFasL	soluble Fas Ligand
SN	supernatant
SSC	Side Scatter (granularity of cells)
ss DNA	single-stranded DNA
TAE	Tris(hydroxymethyl)aminoethane
TNF	Tumor Necrosis Factor
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R	TNF-related apoptosis inducing ligand receptor
TUNEL	TdT-mediated end labeling of DNA

35

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